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STUDY ON THE PHYSIOLOGICAL SIGNIFICANCE OF ENDOTOXIN IN HEATSTROKE

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Cytokines are signaling proteins that regulate immune system function. Though local inflammatory reactions mediated by TNF-a and IL-1b are relatively benign, systemic release of these cytokines can result in vascular shock. It is through such an immune system mechanism that bacterial E upon entering the blood stream evokes vascular shock. E, TNF-a and IL-1b circulate in human heatstroke to suggest E release from a heat-damaged gut may stimulate cytokines that contribute to the vascular shock noted in heatstroke. However, when damaged tissue undergoes necrotic cell death, immune activation can occur with the release of cytokine. Thus, shock cytokines might be released by heat-induced tissue necrosis, even in the absence of E. To further define the physiological significance of E in heatstroke, this study determined if heat injury of HWB could induce a cytokine response in the absence of E. Using thermoelectric technology, HWB was exposed to temperature modulations that precisely replicated those associated with a 70% rat heatstroke mortality rate. Following heat stress, some HWB cultures were exposed to E at concentrations reported to circulate in human heatstroke, while others remained unexposed. In the presence or absence of prior heat stress, the presence of E stimulated inflammatory cytokine (TNF-a and IL-1b) release, while in E's absence such cytokines were not generated. Similar findings were noted for IL-6, a cytokine noted for its modulatory effects on TNF metabolism. Relative to anti-inflammatory cytokines, heat stress and/or E had no effect on IL-1srII, while heat stress reduced E-mediated IL-1ra levels. Thus, some factor, like endotoxin was required to provoke HWB cytokine expression, which tended to support an etiological role for E in heatstroke cytokine responses. Moreover, the heat stress and E conditions that characterize heatstroke affected HWB cytokine metabolism to favor a pro-inflammatory environment.

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BACKGROUND

Immune dysfunction contributes to heat stress injury (27). Endotoxin (E; 2,3) and the immune cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) circulate in human heatstroke (2,3,6,8). These cytokines are also observed during endotoxic shock (15,21) and induce vascular shock in the absence of E (25,28). Thus, a possible scenario for shock in heatstroke is E release from a heat-damaged gut, which leads to the generation of cytokines that mediate, in part, vascular collapse. However, when E's participation is excluded by *in vitro* conditions, injury of artificial human skin by extreme heat stress (90 mins at maximum temperature ≥44°C) results in cytokine expression (4). Though this exceeds conditions normally noted in heatstroke, it suggests a potential for cytokine expression by the direct effects of heat-induced cellular damage, which might occur even in the absence of E. The purpose of this study was to determine if in the absence of E, heat stress conditions like that noted in heatstroke could induce cytokine responses. Such a study might provide insight to the physiological significance of E in heatstroke. The experimental designed required control over E; as such *in vitro* conditions were employed, because *in vivo* systems inherently are plagued by the presence of endogenous gut endotoxins. The *in* vitro system used human whole blood (HWB) to study heat stress effects on inflammatory cytokine, TNF-α, IL-1β expression in the presence or absence of E concentrations noted in human heatstroke (3). A cytokine, interleukin-6 (IL-6) that modulates TNF metabolism was also included for study, as well as the anti-inflammatory cytokines, interleukin-1 receptor antagonist (IL-1ra) and interleukin-1 soluble receptor II (IL-1srII), since anti-inflammatory cytokine pretreatment is protective in rat heatstroke (7,16). The hyperthermic exposures used thermoelectric technology (11) to provide precise control over temperature modulations such that the heat stress replicated those of laboratory animals with a known heatstroke mortality rate (10).

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The views, opinions and/or findings contained in this report are those of the authors and should not be construed as official, policy or decision of the U.S. Army unless so designated by official documentation. For the protection of human subjects, the investigators adhered to policies of applicable Federal Law CFR 46.

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EXECUTIVE SUMMARY

Cytokines are signaling proteins that regulate immune system function. Though local inflammatory reactions mediated by TNF-α and IL-1β are relatively benign, systemic release of these cytokines can result in vascular shock. It is through such an immune system mechanism that bacterial E upon entering the blood stream evoke vascular shock. E, TNF-α and IL-1β circulate in human heatstroke to suggest E release from a heat-damaged gut may stimulate cytokines that contribute to the vascular shock noted in heatstroke. However, when damaged tissue undergoes necrotic cell death, immune activation can occur with the release of cytokine. Thus, shock cytokines might be released by heat-induced tissue necrosis, even in the absence of E. To further define the physiological significance of E in heatstroke, this study determined if heat injury of HWB could induce a cytokine response in the absence of E. Using thermoelectric technology, HWB was exposed to temperature modulations that precisely replicated those associated with a 70% rat heatstroke mortality rate. Following heat stress, some HWB cultures were exposed to E at concentrations reported to circulate in human heatstroke, while others remained unexposed. In the presence or absence of prior heat stress, the presence of E stimulated inflammatory cytokine (TNF-α and IL-1β) release, while in E's absence such cytokines were not generated. Similar findings were noted for IL-6, a cytokine noted for its modulatory effects on TNF metabolism. Relative to anti-inflammatory cytokines, heat stress and/or E had no effect on IL-1srII, while heat stress reduced E-mediated IL-1ra levels. Thus, some factor, like endotoxin was required to provoke HWB cytokine expression, which tended to support an etiological role for E in heatstroke cytokine reponses. Moreover, the heat stress and E conditions that characterize heatstroke affected HWB cytokine metabolism to favor a pro-inflammatory environment.

INTRODUCTION

In the military, heat stress is a concern in training and plays a strategic role in military operations (5,14). Passive or active heatstroke, as occurs in the elderly or those performing physical exertion in hot environments, respectively is also a public health issue (22,23). Evidence of the greenhouse effect and global warming increase concern that the incidence of heatstroke may increase (20,23). Heatstroke prevention and/or treatment require knowledge of the complex factors that contribute to this form of shock. The presence of E and cytokines suggest gut E may play an etiological role in heatstroke (2,3,6,8). However, preventive measures directed solely at E may be ineffective if heat-induced tissue necrosis stimulates cytokine responses in the absence of E. Thus, the physiological significance of E in heatstroke needs to be defined further. In addition, little is know of heat stress and/or E effects on anti-inflammatory cytokines. Using *in vitro* conditions to control for E's presence, the influence of heat stress similar to that in heatstroke was studied to determine if E's presence was required to provoke HWB cytokine responses.

METHODS

After providing informed consent, 10 male subjects donated heparinized (40 units of endotoxin-free heparin/ml) blood samples. Subjects were between 18 and 35 years of age and were non-consumers of tobacco products. They had not experienced body temperature elevations, ill health, wound or body inflammation, and were free of anti-inflammatory drugs for at least one week prior to the blood donation.

Eight mls of the subject's HWB were added to each of two T-250 flasks (Falcon, Becton Dickinson, Oxnard, CA). As previously described (Fig. 1; 11), a thermoelectric module, sandwiched between a heat dispersion plate and a liquid loop heat sink was affixed below each flask. A computer-controlled Series 3 TC^2 thermoelectric cooler controller (Alpha Omega Insts., Norton, MA) directed the thermoelectric module to elevate incrementally the HWB temperature to a desired maximum, maintain this maximum for a desired period of time and then cool the HWB to normal body

temperature in a step-wise fashion. The heat stress was conducted in a CO_2 incubator, such that a 5% CO_2 level could be maintained. As illustrated in Figure 2, maximum temperature and thermal area defined the HWB heat stress and were used to relate the heat stress to heatstroke mortality rate in Sprague-Dawley rats (10).

Following heat stress, one of the heated aliquots of HWB was spiked with E (Escherichia coli 0111:B4, lipopolysaccaride; acetone powder; Sigma-Aldrich, St. Louis MO) at a concentration (10 ng/ml) reported to circulate in human heatstroke (3). This, along with the other heated aliquot, an aliquot exposed only to E and an aliquot left unexposed to heat or E were incubated for 24 hrs under rocking conditions, 5% CO_2 and 37°C. Samples for cytokine and monocyte viability testing were collected at 4 and 24 hrs. Samples for mRNA analysis were collected at 2, 4 and 24 hours.

Enzyme-linked immunosorbant-based assay systems (Endogen, Cambridge, MA) with sensitivities of 10 pg/ml and 1 antomole/reaction were employed in the measurement of cytokine proteins (TNF-α, IL-1β, IL-6, IL-1ra and IL-1srII) and mRNAs (TNF-α and IL-1β), respectively. Extraction of mRNA from white blood cells of the HWB samples employed Tri-reagent (Sigma-Aldrich, St. Louis, MO). The assay system required extracted total RNA to be between 0.047 and 0.75 µg/ml.

Monocyte viability was determined by exposure of 100µl of HWB for 15 mins to ethidium (0.1 µM; Molecular Probes, Eugene, OR) and phycoerythrin-labeled CD14 (20 µl; Becton Dickinson, San Jose, CA). Red blood cells were lysed (FACS lysing solution, Becton Dickinson, San Jose, CA) and the sample analyzed by flow cytometry (Coulter Epics IV, Miami FL). The differential CD14 binding capacity and light side-scatter pattern of white blood cells were used to select a monocyte gate. Presence of ethidium in the nucleus indicated monocyte mortality for the cells within this gate. The percent ethidium positive cells in the monocyte gate were determined.

All assays were duplicated and mean values determined for HWB samples subjected to the presence of prior heat stress (PPHS), PPHS followed by E exposure (PPHS+E), absence of prior heat stress (APHS) or APHS followed by E exposure (APHS+E). A two-way analysis of variance with repeated measures (blood treatment x maximum cytokine, or percent ethidium positive monocytes) was employed to evaluate the data. A Tukey post hoc analysis was used when significant effects were noted. Level of significance was set at $p<0.05$.

RESULTS

Figure 2 illustrates a typical pattern in the degree and temporal temperature changes in the HWB samples using thermoelectric technology (Fig. 1). The heat stress parameters of the HWB donated by the 10 volunteers are shown in Table 1.

PPHS and PPHS+E followed by 24h incubation at 37°C of HWB resulted in percent ethidium positive monocyte increases that were not significantly elevated from time zero (Fig. 3). APHS and APHS+E also showed no significant increases in ethidium positive monocytes after 24h at 37°C.

As illustrated in figure 4, TNF-α concentrations remained undetectable for HWB cultures in the APHS or PPHS. TNF-α was significantly increased for cultures in the APHS+E or PPHS+E. Although not obtaining a point of significant difference at 4 hrs, TNF-α level for HWB cultures in the PPHS+E tended to be elevated above those in the APHS+E. Findings were similar for IL-1β (Fig. 5) and IL-6 (Fig. 6) in that only E-treated cultures showed significant elevations. However, though not significantly elevated, there was a trend for E-treated HWB cultures in the PPHS to have higher IL-1β and IL-6 levels.

Presence of mRNA for TNF-α (2h) and IL-1β (4h) was only noted at low levels for those HWB cultures exposed to E (Table 2). The range of total RNA (0.135 to 0.341 μg) employed was within the limits of the assay system (0.047 to 0.75 μg). No mRNA for TNF-α and IL-1β were detected at time zero and levels at 24h were either undetectable (TNF-α) or decreased (IL-1β).

As illustrated in Figure 7, none of the experimental HWB conditions altered plasma levels of IL-1srII. While HWB in the APHS+E significantly elevated IL-1ra expression, this expression was significantly decreased when HWB was in the PPHS+E (Fig. 8). IL-1ra levels for HWB in the PPHS+E, while not significantly elevated above the PPHS values were significantly elevated above the APHS values. No significant differences were noted in comparisons between PPHS and APHS IL-1ra values.

DISCUSSION

Thermoelectric technology (Fig. 1) permitted heating conditions that precisely replicated the degree and temporal temperature modulations of rat heatstroke. The mean HWB thermal area (Table 1) was equivalent to a 70% heatstroke mortality rate in Sprague-Dawley rats (10). The use of rat heatstroke conditions was necessary, since data relating maximum core temperature and thermal area to human heatstroke mortality rate are not available. Comparisons to rat heatstroke could be criticized, since rats and humans employ different mechanisms to regulate body temperature. However, the mean maximum temperature (Table 1) to which the HWB samples were exposed was greater than the mean rectal temperatures (42.1±0.2°C) reported from human heatstroke victims presenting with endotoxemia and circulating inflammatory cytokines (3). Thus, the conditions employed in this study were relevant to those associated with heatstroke in both rats and humans to indicate the HWB was subjected to a significant heat stress challenge.

As previously demonstrated (9,24), the *in vitro* culture of HWB retains the capacity to elicit a cytokine response when exposed to E (Figs. 4-6). However in the absence of E, temperatures that induce highly lethal rat heatstroke, and endotoxemia and cytokines in human heatstroke did not evoked HWB cytokine responses (Figs. 4-8). This did not appear to be due to a loss in cell viability, since cytokine levels increased over the 24 h test period for cultures exposed to heat stress and E (Figs. 5 and 6). Also, monocyte

mortality as measured by ethidium positive nuclei was not excessive 24 h after heat stress (Fig. 3).

When heat stress is administered simultaneously or preceding E exposure, TNF expression by isolated human monocytes or murine macrophages is depressed (13,17). In contrast, the present study demonstrated PPHS followed by E exposure of HWB did not reduce TNF-α, IL-1β or IL-6 responses (Figs. 4-6). That prior heat stress does not diminish the TNF response to E in rats (1), suggested the current experimental design using HWB more closely reflected the *in vivo* state than studies employing isolated immune cells.

TNF-α and IL-1β mRNA expressions were not induced by HWB in the PPHS, except for when E was present (Table 2). The extracted total RNA employed was within the range required for the assay system. However when E was present, mRNA levels were low. Considering the cytokine levels produced in this study (Figs 3 and 4), this was an unexpected finding. Thus, it cannot be ruled out that the assay sensitivity within the range of total RNA employed may not be sufficient to demonstrate mRNA priming by the PPHS in the absence of E. Moreover, the kinetics of cytokine mRNA metabolism induced by heat stress may be unlike that induced by E and require sampling periods that differed from those employed in this study.

The lack of a cytokine response by heat stress alone may relate to the influences of antigen presenting dendritic cell type (12,19,26) and intensity of antigen interaction with the T-cell receptor (18) on immune responses. As such, HWB under *in vitro* conditions and in the absence of other tissue dendritic cell types might not adequately support the antigen processing and immune cell interactions required for a cytokine response by heat stress in the absence of a strong immune activator, like E. However, human safety and ethical considerations, and the need for control over E necessitated an *in vitro* approach. Similarly, an animal model would have had the inherent problem of endogenous gut E to make control over E's presence or absence problematic.

Another potential contributor to the lack of a cytokine response by heat stress in the absence of E is the issue of blood cell apoptosis. It would seem logical blood cells

would follow an apoptotic pathway of cell death, since this would reduce systemic inflammatory reactions in the advent of significant blood cell injury. If blood cell death inherently followed and apoptotic rather than necrotic form of cell death, then *in vitro* heat-stress of HWB would not be expected to generate a cytokine response. The issue of apoptotic- versus necrotic-mediated forms of blood cell death after heat stress is in need of study.

IL-1srII was unaffected by PPHS and/or E (Fig. 7) to suggest this anti-inflammatory agent does not respond to factors associated with human heatstroke. Unlike with the inflammatory cytokines, TNF-α and IL-1β (Fig. 4-5), PPHS significantly reduced E-mediated IL-1ra, anti-inflammatory cytokine release (Fig. 8). Though other anti-inflammatory agents require study, the finding of reduced E-mediated IL-1ra by PPHS may have important pathophysiological implications, when viewed in light of the reported protective effect of IL-1ra in heatstroke (7,16). Moreover, the present *in vitro* findings perhaps offers an explanation for the protective efficacy of IL-1ra in heatstroke under *in vivo* conditions. However, these findings were in contrast to those of Chang (6) in which no heat stress effect on IL-1ra is noted. Their study employed IgG as the stimulus, which was applied to monocytes isolated at the time of hospital discharge of fully recovered human heatstroke patients. Differences in stimuli and timing of exposure after heat stress may contribute to the conflicting findings. In addition, isolated monocytes as opposed to those in HWB may respond differently. Since E circulates in heatstroke (2,3), its use rather than IgG and the use of HWB rather than isolated immune cells may more closely reflect *in vivo* conditions. Finally, an effect of heat stress on E-mediated IL-1ra expression may have been revealed, since unlike the Chang study (6) that employed normal non-heat-stressed controls, comparisons in the present study were made to a subject's blood sample in the absence of heat stress.

CONCLUSIONS

In the absence of E, HWB heat stress that mirrored the temperature changes of highly lethal rat heatstroke and reached maximum temperatures that exceeded those reported in human heatstroke failed to induce cytokine increases. When E was present at concentrations reported in heatstroke, HWB responded with significant pro-

inflammatory cytokine elevations. As such, some factor normally absent under *in vitro* conditions, like E was required to stimulate HWB cytokine responses after heat stress. This tended to support a physiological role for E in heatstroke cytokine expression. Finally, the conditions of heat stress and E noted in heatstroke influenced *in vitro* inflammatory (TNF-α and Il-1β) and anti-inflammatory (IL-1ra and IL-1srII) cytokines in a manner that would seem to favor pro-inflammatory activity, which may explain why *in vivo* pre-treatment with an anti-inflammatory cytokine (IL-1ra) is protective in heatstroke.

RECOMMENDATIONS

Studies should be designed to define the type of blood cell death (necrotic and/or apoptotic) induced by heat injury. Moreover, since *in vitro* methods are required to control for E's presence or absence in heat-related studies of immune system disturbances, it is necessary to develop models that are more reflective of the complexities of the immune system. As such, an *in vitro* system that permits input from tissues, like the endothelium, skin and/or liver in addition to blood might provide further insight to the influence of heat stress on immune system responses.

Figure 1. Illustration of the thermoelectric system for the regulation of temperature changes in the heating of human whole blood (HWB).

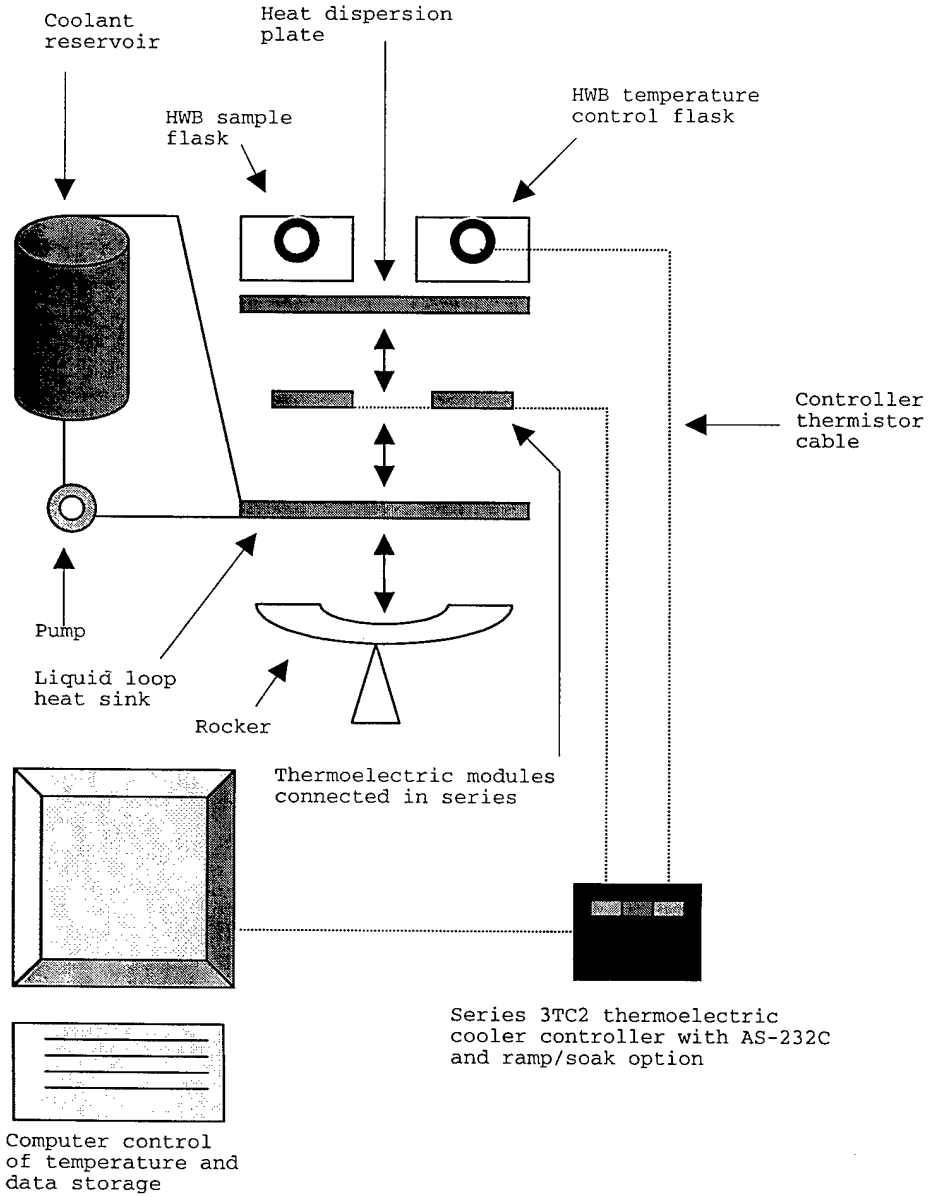
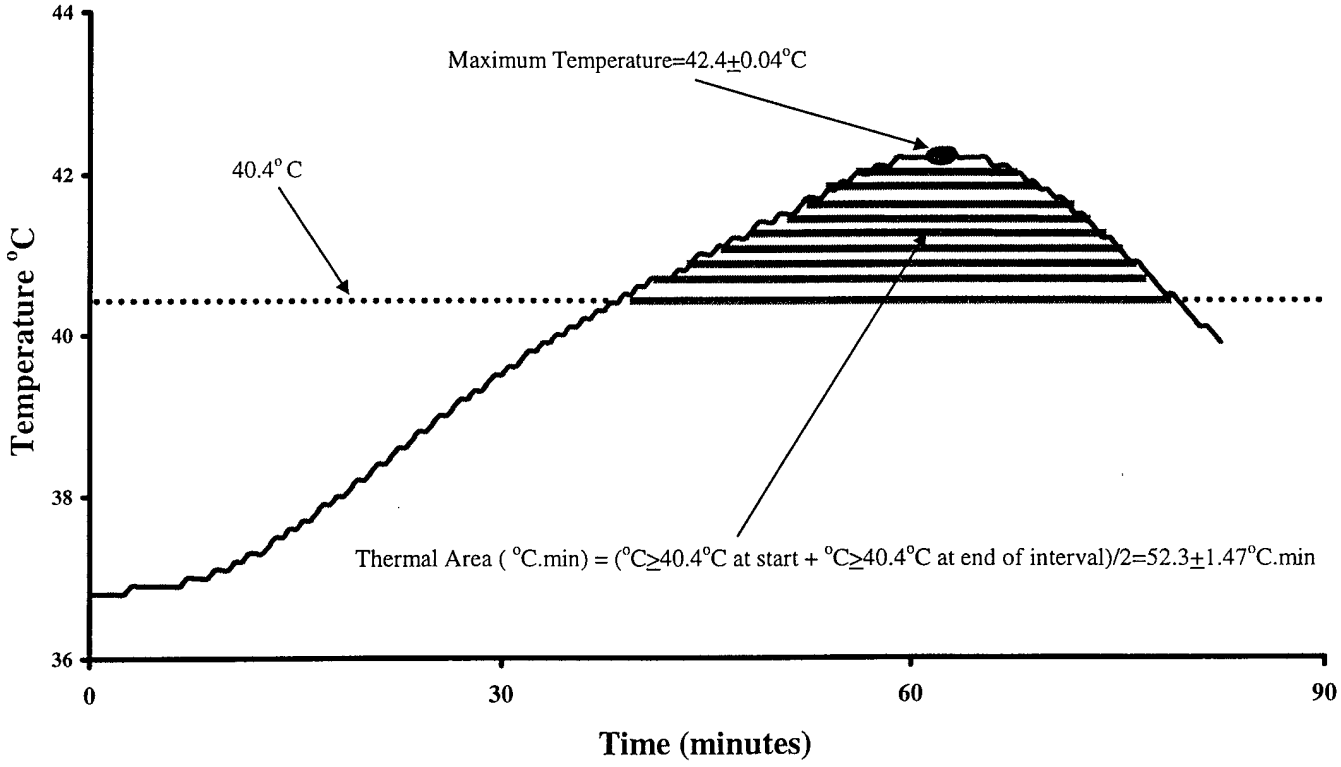
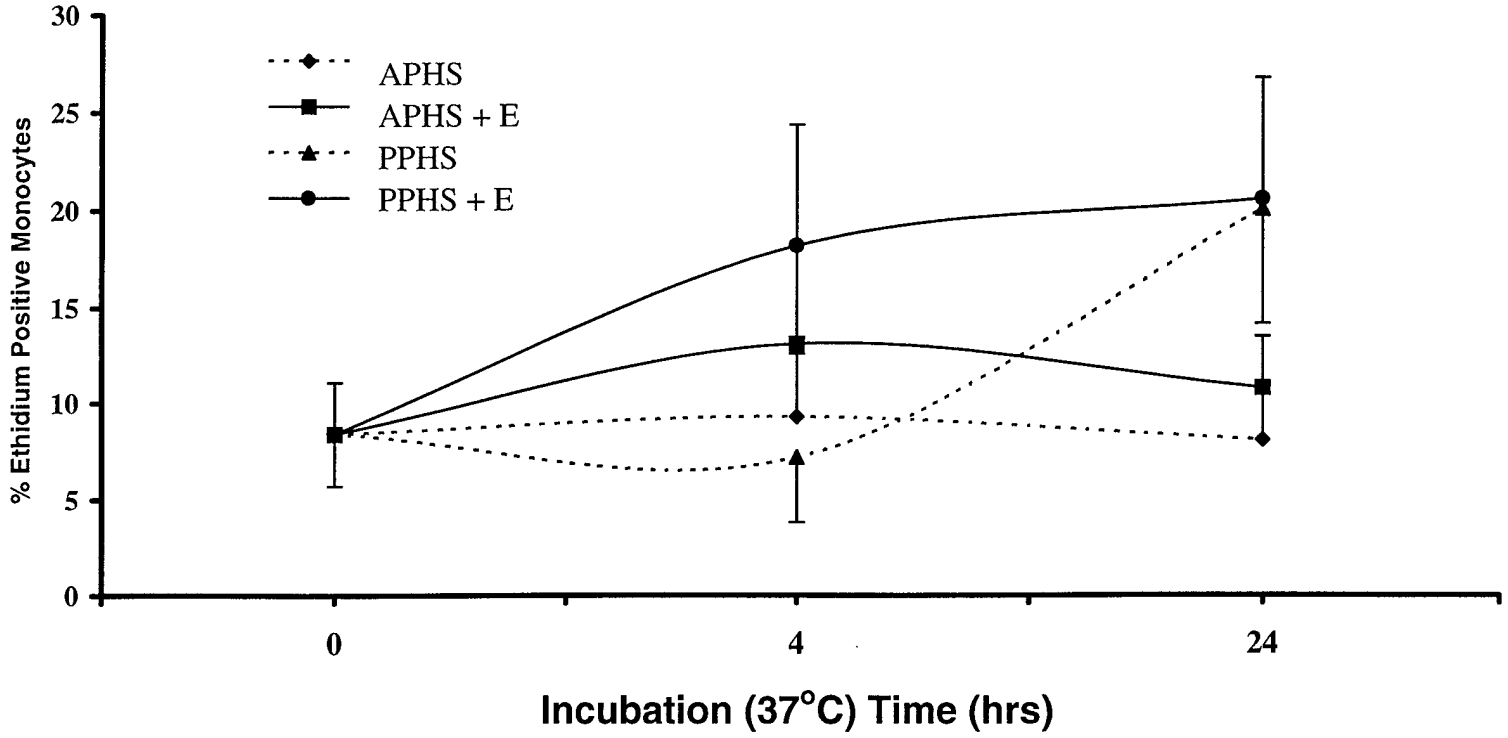


Figure 2. Typical temperature modulation curve for the hyperthermic exposure of human whole blood with an illustration of maximum temperature and calculation of thermal area.



This maximum temperature and thermal area result in a 70% heatstroke mortality rate in Sprague-Dawley rats (10).

Figure 3. Percent of monocytes with ethidium positive nuclei after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).



Prior heat stress involved exposure of HWB to a maximum temperature of 42.4±0.04°C and a thermal area of 52.3±1.47 deg-min. This is a heat exposure that results in a 70% heat stress mortality rate in Sprague-Dawley rats (10). Each point represents the mean ± S.E. determined for HWB samples from ten human volunteers. The inability to exclude ethidium from the cell nucleus is indicative of monocyte mortality.

Figure 4. Changes in tumor necrosis-α levels after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).

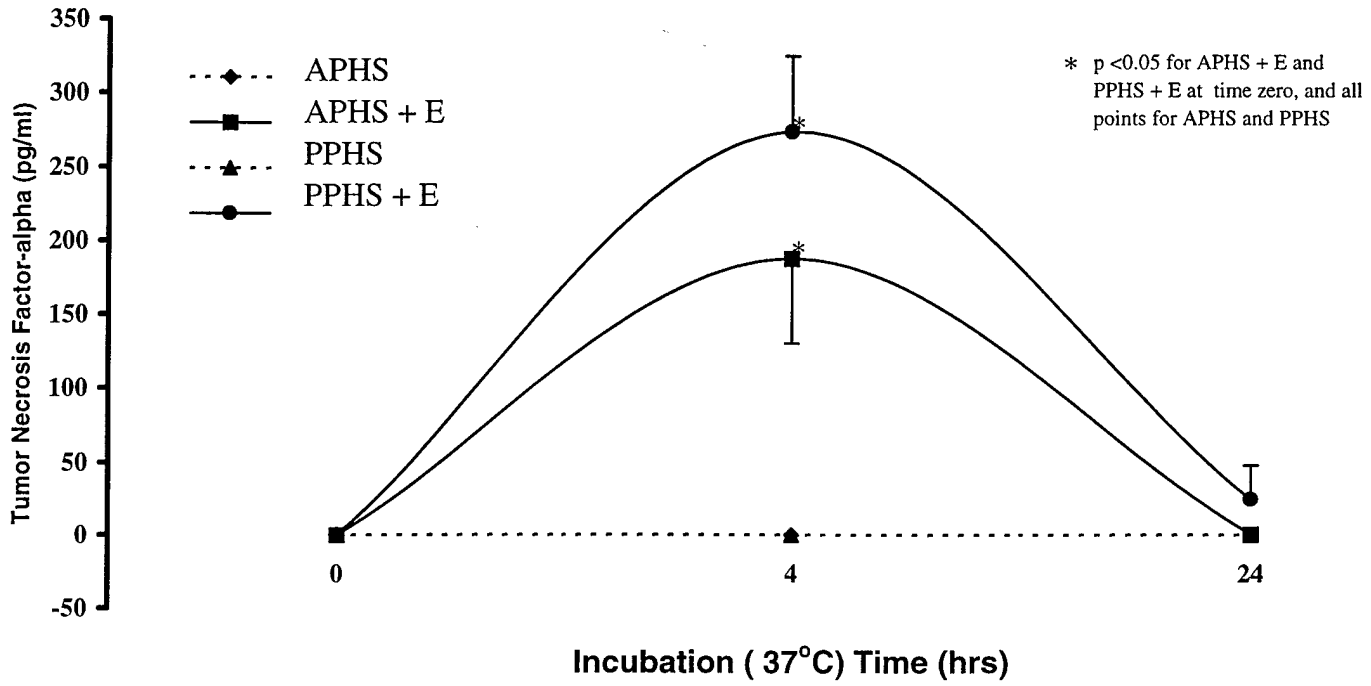


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Figure 5. Changes in interleukin-1β levels after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).

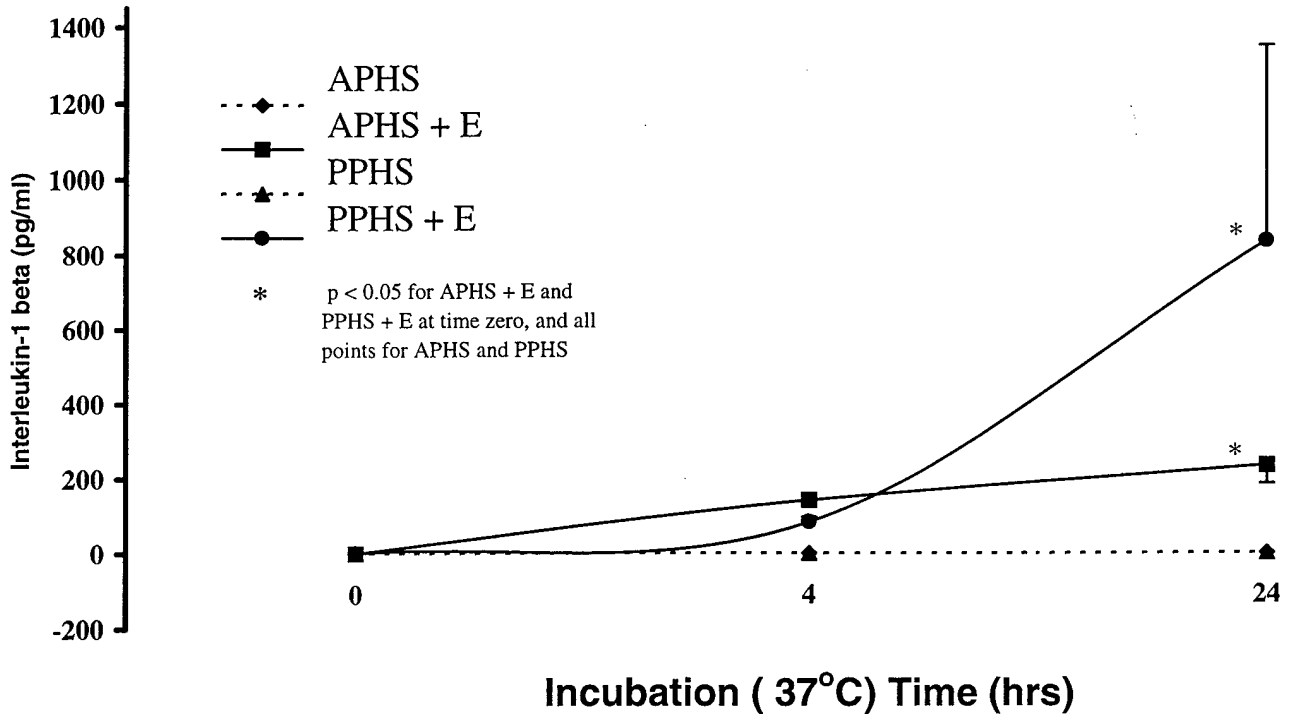

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Figure 6. Changes in interleukin-6 levels after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).

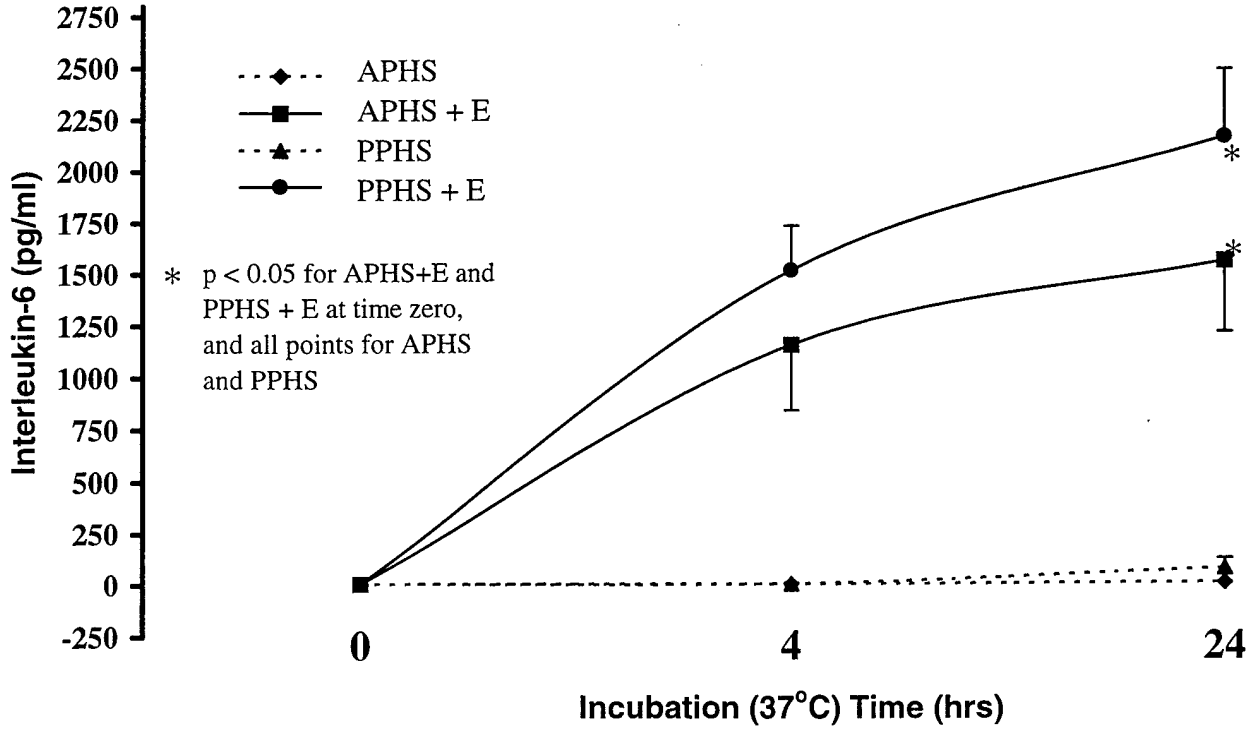

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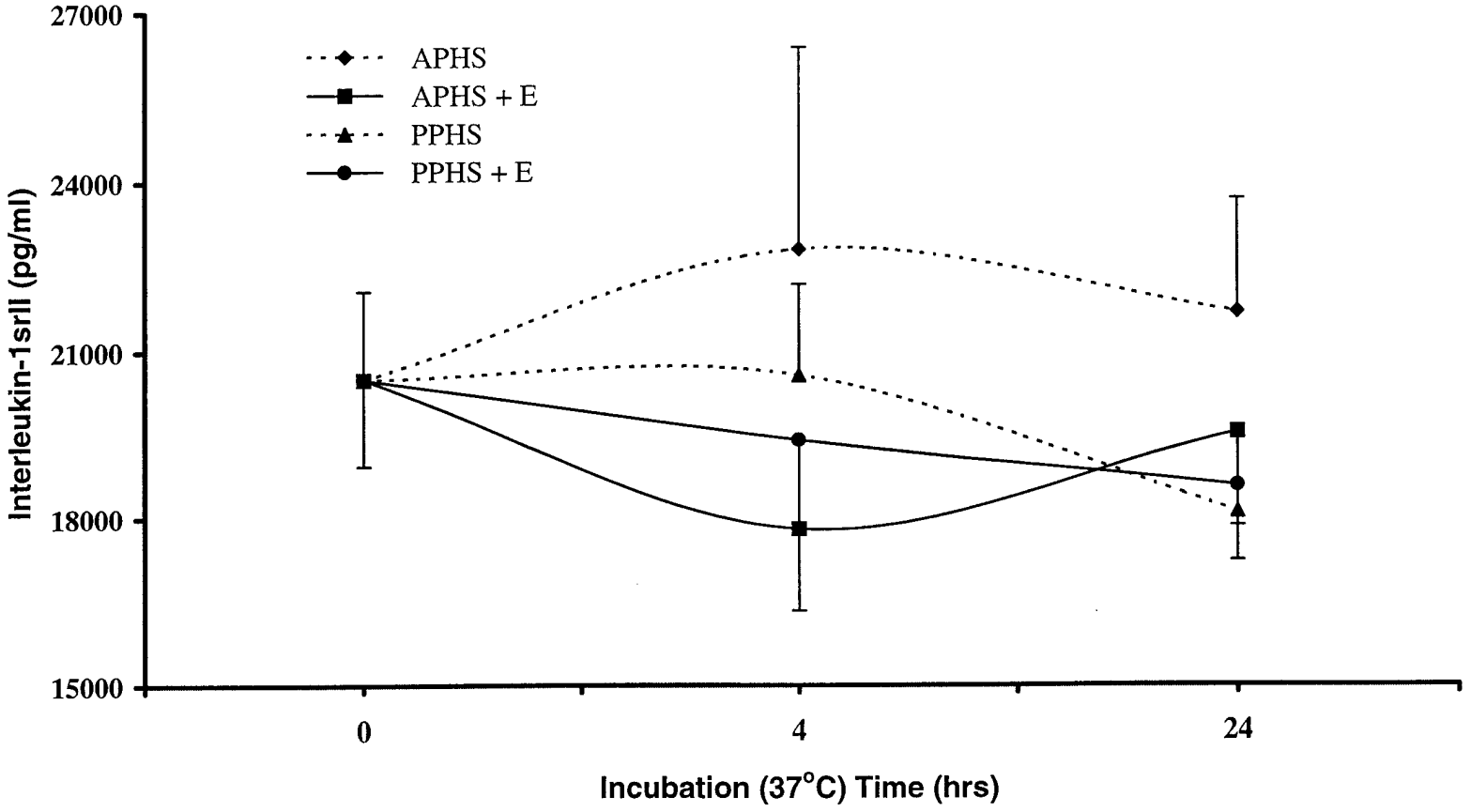
Figure 7. Changes in interleukin-1 soluble receptor II levels after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).



Prior heat stress involved exposure of HWB to a maximum temperature of 42.4±0.04°C and a thermal area of 52.3±1.47 deg-min. This is a heat exposure that results in a 70% heat stress mortality rate in Sprague-Dawley rats (10). Each point represents the mean ± S.E. determined for HWB samples from ten human volunteers.

Figure 8. Changes in interleukin-1 receptor antagonist levels after subjection of human whole blood (HWB) to the presence (PPHS) or absence (APHS) of prior heat stress followed by HWB incubation (37°C) in the presence (+E) or absence of bacterial endotoxin (10ng/ml).

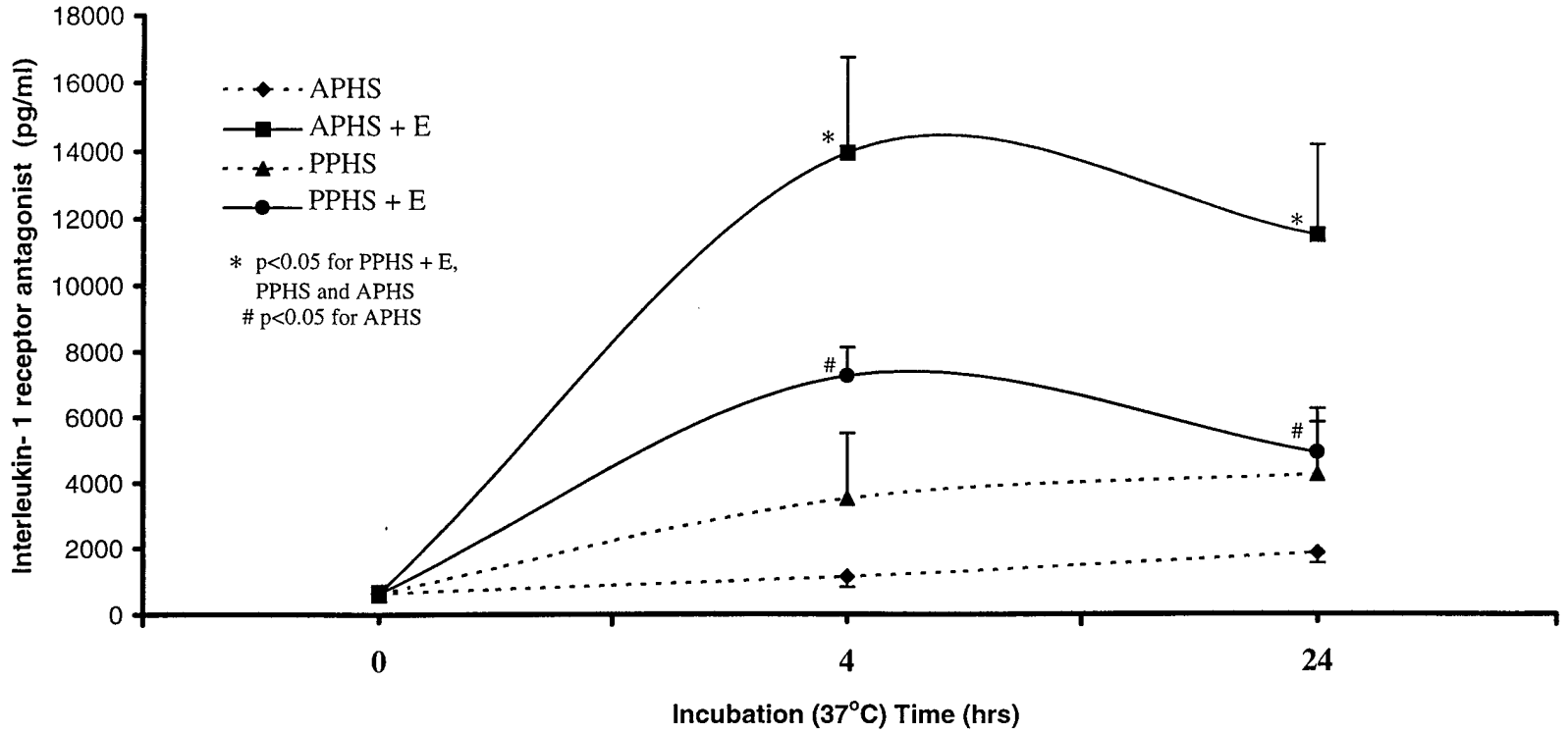


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Table 1. Human Whole Blood Heat Stress Parameters.

	Max. Temp. (°C)	Time at Max. Temp. (mins)	Thermal Area (°C.min)
Mean	42.4	6.3	52.3
±S.E.	0.04	0.67	1.47

These parameters of heat stress result in a 70% mortality rate in Sprague-Dawley Rats (10).

Table 2. Influence of the presence (PPHS) or absence (APHS) of prior heat stress and/or endotoxin (+E) on human whole blood (HWB) tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) messenger RNA (mRNA; attomoles/reaction) expression relative to total RNA (tRNA; μg) extracted from white blood cells in the HWB samples.

	APHS mRNA	(tRNA)	**PPHS+E mRNA**	(tRNA)	**PPHS mRNA**	(tRNA)	**PPHS+E mRNA**	(tRNA)
TNF-α	**0.00**	(0.299)	**0.700**	(0.276)	**0.00**	(0.207)	**0.600**	(0.245)
[2h*]	**±0.00**	(±0.054)	**±0.540**	(±0.026)	**±0.00**	(±0.011)	**±0.303**	(±0.032)
IL-1β	**0.00**	(0.135)	**1.700**	(0.341)	**0.00**	(0.335)	**2.90**	(0.264)
[4h*]	**±0.00**	(±0.042)	**±0.306**	(±0.026)	**±0.00**	(±0.080)	**±0.538**	(±0.030)

*= length of 37°C incubation after exposure to heat stress and/or E; mRNA for TNF-α or IL-1β was not noted at time zero, while after 24h of incubation mRNA for TNF-α was rarely detected and mean values for IL-1β were reduced from those determined at 4 h.

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